IN VITRO ASSESSMENT OF DENDRITIC CELL ACTIVATION AND PHENOTYPIC RESPONSE TO GOLD NANOPARTICLE TREATMENTS

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Ethan E. Jordan

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IN VITRO ASSESSMENT OF DENDRITIC CELL ACTIVATION AND PHENOTYPIC RESPONSE TO GOLD NANOPARTICLE TREATMENTS

Approved by:

Dr. Julia Babensee, Advisor
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Balakrishna Pai
Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Esfandiar Behravesh
Department of Biomedical Engineering
Georgia Institute of Technology

Date Approved: 03 May 2019

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CONFLICTS OF INTEREST

I would like to acknowledge that the research I have conducted in the Babensee Lab regarding dendritic cells and nanoparticles at the Georgia Institute of Technology is a continuation of an undergraduate research project started by Nandhini Prakash and Christina Koutrelakos. The background materials, as well as the protocol, methods developed to conduct the experiment, and a fraction of the data collected were generated by them before I took over. I would also like to acknowledge Peng Meng Kao, Julia Babensee, and Sangeetha Srinivasan for their work, and its contribution to the fulfillment of my degree. I claim no conflicts of interest and want to acknowledge everyone that has contributed to this body of research that has been conducted.

LIST OF ABBREVIATIONS

Ab: antibody

APC: antigen presenting cell

AuNP: gold nanoparticle

CD86: cluster of differentiation 86

DC: dendritic cells

DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non integrin GM-

CSF: granulocyte-macrophage colony-stimulating factor

iDC: immature dendritic cell

IMF: inflammatory maturation factor

IL: interleukin

ILT3: immunoglobulin-like transcript 3

LPS: lipopolysaccharide

mDC: mature dendritic cell

MHC: major histocompatibility complex

NP: nanoparticle

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PEG: polyethylene glycol

tDC: tolerogenic DCs

TMF: tolerogenic maturation factor

TNF: tumor necrosis factor

Tregs: regulatory T-cells

I. Abstract [41]

Dendritic Cells (DCs) have shown great potential in a variety of immunotherapeutic applications due to their key role in both the innate and adaptive immune response. This study aims to observe the activation and phenotypic changes within the cell population following treatment with a variety of gold nanoparticles (AuNPs).

AuNPs are currently being used as drug delivery vehicles, regulators and suppressors of the host immune response. They have easily modified surface chemistry and display high biocompatibility.

Developing mechanisms to specifically manipulate DCs utilizing biomaterials will allow for a number of immunotherapeutic approaches to become available to patients suffering from autoimmune diseases such as HIV/AIDS, and cancer.

Following treatment with numerous AuNP treatments varying in surface coating and concentration cells should demonstrate no abnormal death or patterns within the cell cycle and should also exhibit elevation in surface markers correlating to tolerization.

II. Introduction

Nanoparticles (NPs) have become an increasingly investigated subject within the scientific community due to their high potential as a treatment option for cancer and numerous other diseases originating within and impacting the immune system. Research continues to broaden as investigators attempt to obtain a clearer understanding of NP characteristics and how to most effectively utilize their immunotherapeutic potentials.

NPs demonstrate functionality in many applications such as drug delivery and release, immune response modulation, and diagnostic imaging. NP characteristics that are of interest are material, size, shape, and physicochemical surface alterations such as PEGylation or autologous serum-based coating. These specific characteristics exhibit precise control over NP function, and the research being conducted within this proposal specifically explores NP uptake by dendritic cells (DCs) and the subsequent impact NP treatments have on the activation state and phenotypic responses of DCs. DCs are antigen presenting cells (APCs) that play a distinct role in both the innate and adaptive immune response. An area still requiring further study is the impact that NPs could potentially have on the phenotypic profiles seen throughout the DC differentiation process.

Gold nanoparticle (AuNP) samples have been prepared in the Wilhelm Lab at the University of Oklahoma in different sizes and coatings that will allow for manipulation and observation of DC phenotype. Maturation and tolerogenic markers were both fluorescently labeled to quantify expression following NP treatment. Fluorescent labelling has also been used to normalize

collected data to the proportion of the cell population consisting only of DCs. Observing phenotypic trends will allow for further development and improvement of biomaterial systems to modulate host immune response with increased efficacy. Ultimately, this work is expected to result in the development of therapeutic treatment options for patients suffering from immunological diseases.

Literature Review [41]

1. Innate and Adaptive immunity

There are two types of immunity that protect every distinction of vertebrates: the innate and adaptive immune systems. The innate immune response acts as the first line of defense against foreign entities which is often achieved by marking foreign entities with activation fragments [1]. These fragments have the potential to be identified by phagocytes for destruction of foreign cells [2, 3]. The adaptive response is composed of a set of plasma proteins that can bind to pathogen surfaces leading to proteolytic cleavage, whose fragments mediate an inflammatory response, recognition of pathogens by phagocytes, and finally results in lysis of the cell via membrane attack complex (MAC) [4, 5]. The recognition of pathogens by these phagocytes results in cytokine and chemokine release, attracting monocytes and neutrophils which will infiltrate infected tissues and initiate the inflammatory response [6].

2. Dendritic Cells

Dendritic Cells (DCs) are antigen presenting cells (APCs) that are activated to mediate a host immune response. They play an integral role in both innate and adaptive immunity via their role in T cell activation. The primary functions of DCs are capturing and presenting antigens and other foreign bodies to other cells within the immune system. In order to accomplish this, DCs phagocytize antigens, process them internally, and present the resulting antigen peptide on their cell surface. DCs are located in nearly all tissues and organs including the lymphoid organs. The contribution of DCs concerning the stimulation of specific T cell responses has shown potential

in developing new vaccine strategies for the treatment of a number of different ailments, including infections, allergic and autoimmune diseases, and cancer [7].

DCs originate from myeloid progenitor cells in the bone marrow which have the ability to differentiate into many different cell types. Monocytes are produced daily, on the order of multiple millions, and normally circulate through the bloodstream for a brief period of time before apoptosing spontaneously [8]. However, during an innate immune response, cytokines and chemokines excreted by leukocytes recruit monocytes to the site of inflammation [9]. At the site of inflammation, the monocytes will permeate the tissues and differentiate into immature dendritic cells (iDCs) when in the presence of granulocytemacrophage colony-stimulating factor (GM- CSF) and interleukin-4 (IL-4) [10]. Initially, chemoattractants resulting from tissue damage, pathogens products, and inflammatory cytokines attract iDCs to the site of infection. iDCs phagocytize antigens, degrade them via endocytosis, and produce antigenic peptides capable of binding to major histocompatibility complex class I (MHC I) and MHC II molecules. Following synthesis and expression of these molecules on the DC cell surface, the phenotype of the cell changes from an iDC to a mature dendritic cell (mDC).

The function of the DC also changes from capturing antigens to presenting them, denoting them as antigen presenting cells (APCs). mDCs lack responsiveness to inflammatory chemokines and demonstrate responsiveness to lymphoid chemokines. Lymphoid chemokines will influence their migration to the draining lymph node [11]. Once they have infiltrated the lymph nodes, DCs conduct physical interactions with T cells to activate, or suppress, the adaptive immune response.

3. **Gold Nanoparticles**

Nanoparticles (NPs) are defined as an object within the range of 1-100nm in size. The function and use of nanoparticles are innumerable with precise control over the material, size, shape, surface chemistry, and treatment concentration. Concerning immunotherapies, gold nanoparticles (AuNPs) have been found to be effective due to their stability, solubility, particle size, and affinity to attach surface molecules [12]. This control can be utilized to allow AuNPs to function as antigen carriers, which can stimulate antigen presentation when phagocytized by iDCs. Using different surface coatings and growth factor treatments the maturation of the iDCs can by controlled and observed throughout the differentiation process. This process is not well understood and requires further revision considering all facets of engineering AuNP treatments. AuNPs have been shown to migrate to the lymph nodes when they are within the 20-200nm range [13]. Within this size range, larger particles demonstrate a decrease in the quantity of captured AuNPs. Lower sizes were also shown to be effective, demonstrating increased expression of proinflammatory genes in conjunction with minimal cytotoxicity when compared to silver NPs. Through numerous optimization trials, the 60nm gold particles were determined to be the most effective compromise between size and material [14, 15, 16].

Though studies have determined the optimal AuNP size, concentration of cell treatments and particle surface alterations require further elucidation. Increasing the concentration of AuNPs have shown correlation with an increase in cytotoxicity, while lower concentrations have shown decreases in the quantity of uptake particles [17, 18]. Additional studies have observed surface chemistry correlations to NP uptake, however, the role of maturation in this process still remains unclear [19]. In order to understand and optimize the process in which iDCs differentiate into either mDCs or tDCs, more research must be done to elucidate the impact of treatment concentration and surface modifications to the particles being used.

Methods and Materials [41]

1. Cell Collection and Isolation

Cells will be collected via whole blood collection at STAMPS Healthcare Center at Georgia Institute of Technology by a certified phlebotomist in accordance with protocol H15072 and associated informed consent documentation.

Following the successful collection of whole blood from a donor, monocytes will be isolated from the sample, washed, and prepared for differentiation through growth factor treatments throughout the culture period. Whole blood will be diluted two-fold with phosphate buffered saline (PBS), and then this diluted mixture will be centrifuged in lymphocyte separation media (LSM). This centrifuge period will spin down the red blood cells from the sample, and leave a supernatant composed of monocytes and plasma components.

2. Counting Cells

Cells will be counted with one of two methods. The most commonly used method within this laboratory is to count a small sample of cells (10uL of cells + media) suspended in Isoton II electrolyte solution. The sample will be analyzed using the Multisizer 4e in the Cellular Analysis and Spectroscopy Core in the Petite institute. If this machine is unavailable, the cells will be counted using conventional hemacytometer techniques with a one-to-one dilution of cell suspension with trypan blue. They are then counted beaneath a microscope and resuspended to the desired concentration after being centrifuged once again.

3. Human Cell Culture

Monocytes isolated from the whole blood draw will be cultured in tissue-culture treated polystyrene plates (5.0E5 cells/mL) in complete RPMI-1640 media containing 10% fetal bovine serum (FBS) and 10% penicillin/streptomyocin (PEN/STREP). The cells will also be treated with Interleukin-4 (IL-4) as well as granulocytemacrophage colony-stimulating factor (GM-CSF) to induce proper differentiation of monocytes into DCs. This treatment with fresh, pre-warmed media, IL-4, and GM-CSF will be repeated on the fifth day of treatment with the preparation of control groups.

Control groups will include iDCs, mDCs, tDCs. To stimulate the DCs to further differentiate into different phenotypes a fraction of the initial population will undergo two different treatments. To stimulate DCs to differentiate into mDCs, cells are treated with LPS (50 U/uL) in conjunction with IL-4 and GM-CSF. Subsequently, tDCs will be stimulated by equal treatment with interleukin-10 (IL-10) and Interferon Alpha (INF-a).

These three sets of controls: iDCs, mDCs. tDCs, will be prepared and transferred and seeded into a sterile 96-well, tissue-culture treated, microplate. The remainder of the wells within the plate will then be seeded with a combination of twelve different biomaterial treatments. These treatments will adjust the surface coating of the biomaterial in conjunction with a range of concentrations.

4. **Human Serum Extraction [41]**

Between day 0 and day 4 of DC culture, whole blood was collected from the same consenting donor as day 0 at STAMPS phlebotomy laboratory in accordance with protocol H10011 of H15072 approved by the IRB at the Georgia Institute of Technology. The blood was allowed to clot for 30 minutes at 25^{o} C and centrifuged at 2000g for 10 minutes. The resultant supernatant

contained a pellet of red blood cells and serum. The serum was extracted manually with an automatic pipette and stored at 0^{o} C until used for NP preparation on day 5.

5. **Gold Nanoparticle Treatment Preparation [41]**

Gold nanoparticles were kindly supplied by the Wilhelm Lab at University of Oklahoma at 60nm and 5nM as stock solutions containing Bare, PEG-2K coated and PEG-5K coated particles. Bare nanoparticles were suspended in ultrapure water and PEGylated AuNPs were suspended in PBS in their respective stock solutions [20].

Bare, PEG-2K and PEG-5K coated AuNP treatment preparation

32 μL of each stock solution were prepared; two samples of the bare AuNP stock were prepared, one for bare AuNP treatment and a second for the serum-coated AuNP treatment group. The four samples were centrifuged for 35 minutes at 1200g to pellet the AuNPs, then each was washed twice with sodium citrate tribasic dehydrate solution (200 μL, 5 mM), centrifuging samples for 35 minutes at 1200g and aspirating the supernatant after each wash. The resulting AuNP pellets of one of the bare AuNP samples, the PEG-2K AuNP aliquot and the PEG-5K AuNP aliquot were resuspended in 1 mL RPMI media, for an AuNP treatment sample concentration of 160 pM. A serial dilution was performed on these three of the aliquots to obtain three concentrations (160 pM, 16 pM, and 1.6 pM) for each AuNP coating type.

Serum-coated AuNP treatment preparation

The pellet in the remaining sample of bare AuNPs was resuspended in 40 μL of chilled PBS, and added to an Eppendorf tube containing 400 μL of human serum (filtered with a 0.22 μm PES filter). The AuNPs were incubated in human serum for 1 hour with 95% relative humidity and

5% CO_2 at 37◦C. After incubation, the serum-AuNP solution was centrifuged for 35 min at 1200g and the supernatant was aspirated. The serum-coated AuNPs were washed with chilled PBST twice by adding 750 ul of chilled PBST, centrifuging for 35 min at 1200g, and aspirating the supernatant. After the second PBST wash, the serum-coated AuNPs were washed with 1 mL chilled PBS by the same process. The final serum-coated AuNP pellet was resuspended in 1 mL RPMI media, for an AuNP treatment sample concentration of 160 pM. A serial dilution was performed to obtain three concentrations:160 pM, 16 pM, and 1.6 pM.

6. **Treatment of iDCs with Gold Nanoparticles in HTP Format [41]**

On day 5 of DC culture, NP treatments were prepared as described in section 4.2.3. Loosely adherent and non-adherent iDCs were harvested and resuspended in DC media with 1000 U/ml GM-CSF and 800 U/ml IL-4 at $5x10^5$ DCs/ml. 100 μL of iDCs at a concentration of 5×10^5 DCs/ml were plated onto each well in the 96-well tissue culture plate (Corning). The wells for the negative control of iDCs remained untreated, the wells for the positive control of mDCs were treated with LPS (1 mg/mL; *E. coli* 055:B5; Sigma), and the wells for the positive control of tDCs were treated with human IL-10 (3,500 U/mL; R&D Systems) and human IFN-alpha (35,000 U/mL; R&D Systems). 150 μl of each concentration group for each AuNP coating treatment group were added to different wells of the 96-well plate containing iDCs, such that the final concentration of AuNPs in each well were 0.1 pM, 1.0 pM and 10 pM (Figure 1). The iDCs were then incubated with the control, bare AuNP, PEG-2K coated AuNP, PEG-5K coated AuNP, and serum coated AuNP treatments for 24 hours with 95% relative humidity and 5% CO_2 at 37◦C.

On day 6, the DCs treated in the 96-well plate were transferred directly to wells of a 96-well black filter plate wetted with PBS. The supernatants were removed by centrifuging the filter plate for 4 minutes at 400rpm. To each well, 100 µl of cold working fixation solution (0.05 % paraformaldehyde) was added, and the plate was incubated for at least 30 min at room temperature on a microplate shaker at 450 rpm.

(VWR, West Chester, PA) followed by the removal of the fixative by centrifugation (4 minutes, 400 rpm). Subsequently, DCs were stained with antibodies for surface marker expression, namely, anti-DC-SIGN-FITC (Clone 120507; R & D Systems), anti-CD86- PE (Clone BU63; Ancell) and anti-ILT3-AF647 (Clone ZM4.1, Biolegend). For isotype staining for background fluorescence elimination, the following antibodies were used: IgG2B-FITC (clone 133303; R&D Systems), IgG1-PE (clone MOPC31C; Ancell) and IgG1κ (clone MOPC-21; Biolegend) (Figure 1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Antibody 150 ul/well iDC			Isotyp 150 ul/well iDC			Antibody 60nm - 150 ul 0.1pM Au(PEG5K)			Cells-Back-ground	Cells-Back-ground	Cells-Back-ground
B	Antibody 150 ul/well mDC			Isotyp 150 ul mDC			Isotyp 60nm - 150 ul 0.1pM Au(PEG5K)			Media	Media	Media
C	Antibody 150 ul/well tDC			Isotyp 150 ul tDC			Antibody 60nm - 150 ul 1pM Au(PEG5K)			Isotyp 60nm - 150 ul 1pM Au(PEG5K)		
D	Antibody 60nm - 150 ul 0.1pM Au			Isotyp 60nm - 150 ul 0.1pM Au			Antibody 60nm - 150 ul 10pM Au(PEG5K)			Isotyp 60nm - 150 ul 10pM Au(PEG5K)		
E	Antibody 60nm - 150 ul 1pM Au			Isotyp 60nm - 150 ul 1pM Au			Antibody 60nm - 150 ul 0.1pM Au(PEG2K)			Isotyp 60nm - 150 ul 0.1pM Au(PEG2K)		
F	Antibody 60nm - 150 ul 10pM Au			Isotyp 60nm - 150 ul 10pM Au			Antibody 60nm - 150 ul 1pM Au(PEG2K)			Isotyp 60nm - 150 ul 1pM Au(PEG2K)		
G	Antibody 60nm - 150 ul 0.1pM Au(Serum)			Isotyp 60nm - 150 ul 0.1pM Au(Serum)			Antibody 60nm - 150 ul 10pM Au(PEG2K)			Isotyp 60nm - 150 ul 10pM Au(PEG2K)		
H	Antibody 60nm - 150 ul 1pM Au(Serum)			Isotyp 60nm - 150 ul 1pM Au(Serum)			Antibody 60nm - 150 ul 10pM Au(Serum)			Isotyp 60nm - 150 ul 10pM Au(Serum)		

Figure 1. Layout of AuNP-DC treatments in 96-well plate. AuNP concentrations of 0.1 pM, 1.0 pM and 10 pM were added to DCs in individual wells for all AuNP treatment groups (bare, serum coated, PEG-2K coated, and PEG-5K coated AuNPs). Antibody staining was added to three of the six wells used for each treatment, and isotype staining was added to the other three wells.

The plate was incubated in a plate shaker (600 rpm) at 40oC for 40 minutes protected from light. After this staining procedure, DCs were washed three times with washing solution of 0.1% BSA and 2mM EDTA in PBS, pH 7.20 by centrifugation at 400 RCF for 4 min. Afterwards, 100 µl of the washing solution was added to DCs and the fluorescence of each treatment group were measured. The geometric mean fluorescent intensities were measured with a Tecan Infinite F500 microplate reader (Tecan US, Durham, NC) using excitation filters of 535/25 and 485/20 and the emission filters of 590/20 and 535/25, for PE and FITC, respectively, and 650/668 for anti-ILT3 - AF647.

The surface marker, CD86, is a costimulatory molecule for which expression becomes up-regulated upon DCs maturation. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) is an endocytic receptor which expression is not significantly down-regulated upon DCs maturation. Immunoglobulin- like transcript 3 (ILT3) is an inhibitory receptors which expression is up-regulated upon anti-inflammatory (tolerogenic) DC response. The ratio of respective geometric mean fluorescent intensities for CD86 expression divided by DC-SIGN expression defines the metric of "inflammatory maturation factor" (IMF) as an indicator of pro-inflammatory DC phenotype. The ratio of respective geometric mean fluorescent intensities for ILT3 expression divided by CD86 expression defines the metric of "tolerogenic maturation factor" (TMF) as an indicator of tDC phenotype.

7. **Cellular Analysis**

Cells will be analyzed using fluorescent imagine in a high-throughput format within the Petite Institute at Georgia Tech.

Initially, cells will be prepared for analysis using standard fixation techniques with 4.0% formaldehyde solution prepared in the lab using paraformaldehyde and PBS. This solution is then pH balanced to 7.1. Cells will then be transferred to a 96-well filtration plate, which will allow for treatments and washing cycles to be conducted more efficiently. Following fixation, cells will be stained with an antibody panel that will allow for proper identification of only the DC population (DCSIGN), and then also stained with markers to identify cell populations according to their surface receptors: mDCs (CD86) and tDCs (ILT-3). This staining will happen in a dark environment, while being shaken at 450 RPM at 4^{o} Celsius.

Once cells are stained, they fluorescence intensity will be measured using a micro-plate reader within the lab. This plate reader will measure the fluorescence at nine different locations within each well to account for variability in cell seeding, as well as precise pipetting. The plate reader will take these measurements automatically, and then a statistical analysis will be conducted on the raw data to compare the proportions of each cell type relative to the overall population.

In addition to this analysis, observations will be made to determine the impact of biomaterial treatment with the different coatings and concentrations on the NPs used to treat the DCs. Trends and effective treatments will lead to further modulation of the materials, and further study within *in vivo* models.

Results

DCs Treated with Serum Coated Particles Exhibited Highest Tolerogenic Maturation Expression [41]

The TMF, as defined by the ratio of the geometric mean fluorescent intensities for ILT3/CD86, was determined for each AuNP coating type and concentration. As expected, the DCs treated with IL-10 and IFN-alpha to act as the positive tDC control had a significantly higher TMF than both the iDC and mDC controls, and the mDC control had a very low TMF (Figure 2). The TMF for the tDC control was higher than DCs treated with any concentration of bare, PEG-2K, or PEG-5K coated AuNPs. However, all concentrations of the serum-coated AuNP treatments showed a higher TMF than any other treatment group, including the tDC control. The PEG-5K coated AuNP treatment group of DCs had TMFs that were lower than the iDC control for AuNP concentrations 0.1 pM and 10 pM; at 1 pM, the TMF was higher than that of the iDC control.

The bare AuNP treatment, serum coated AuNP treatment, and PEG-2K treatment showed a slight concentration dependence with increasing concentrations of AuNPs and an increased TMF.

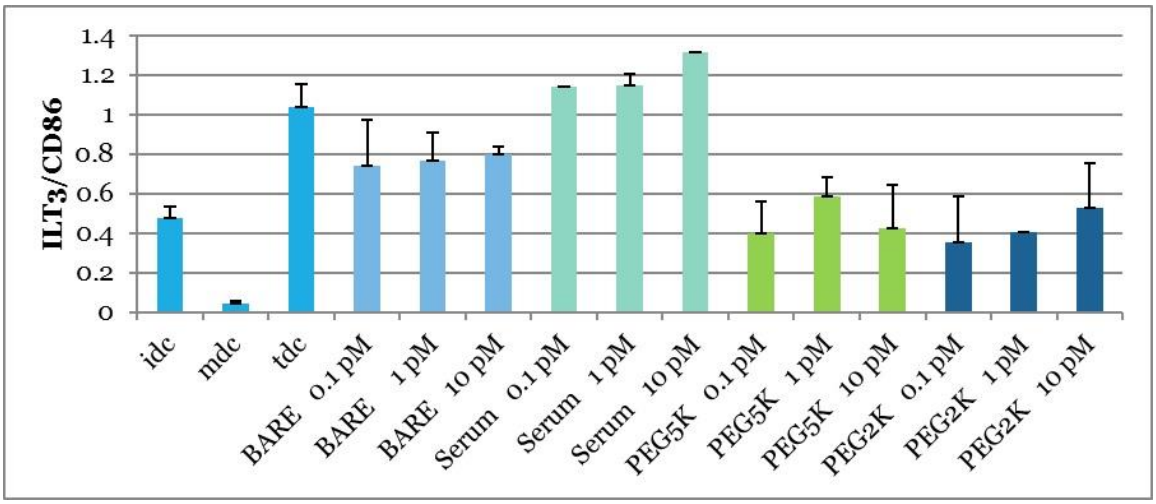


Figure 2. Tolerogenic maturation factor (ILT3/CD86) of DCs treated with AuNPs with mean ± range, n=2 donors. Treatments of different AuNP coatings (bare, serum, PEG-2K, PEG-5K) and concentrations (0.1 pM, 1.0 pM, 10 pM) were used. DCs were incubated with AuNP treatments for 24 hours at 37°C. DCs were stained with antibodies for surface marker expression, stained with isotype for background and geometric mean fluorescent intensities were determined for ILT3 and CD86 expression for each treatment group using a Tecan Infinite F500 microplate reader.

Inflammatory maturation factor expression showed concentration dependence for DCs treated with bare AuNPs and serum-coated AuNPs [41]

The IMF, as defined by the ratio of the geometric mean fluorescent intensities for CD86/DCSIGN, was determined for each AuNP coating type and concentration. As expected, the positive control for inflammatory DC maturation (mDC) had the highest IMF, and the positive control for tolerogenic DC maturation (tDC) had the lowest IMF. DCs treated with the bare AuNP treatment group showed a strong positive correlation with increasing bare AuNP concentration and an increased IMF. The reverse trend was seen with the serum coated AuNP

treatment group, where increasing concentrations of serum coated AuNPs were correlated to a decrease in the IMF. This apparent concentration dependence for the serum coated AuNPs was seen with the TMF as well, with increasing concentrations of AuNPs correlating to an increased TMF.

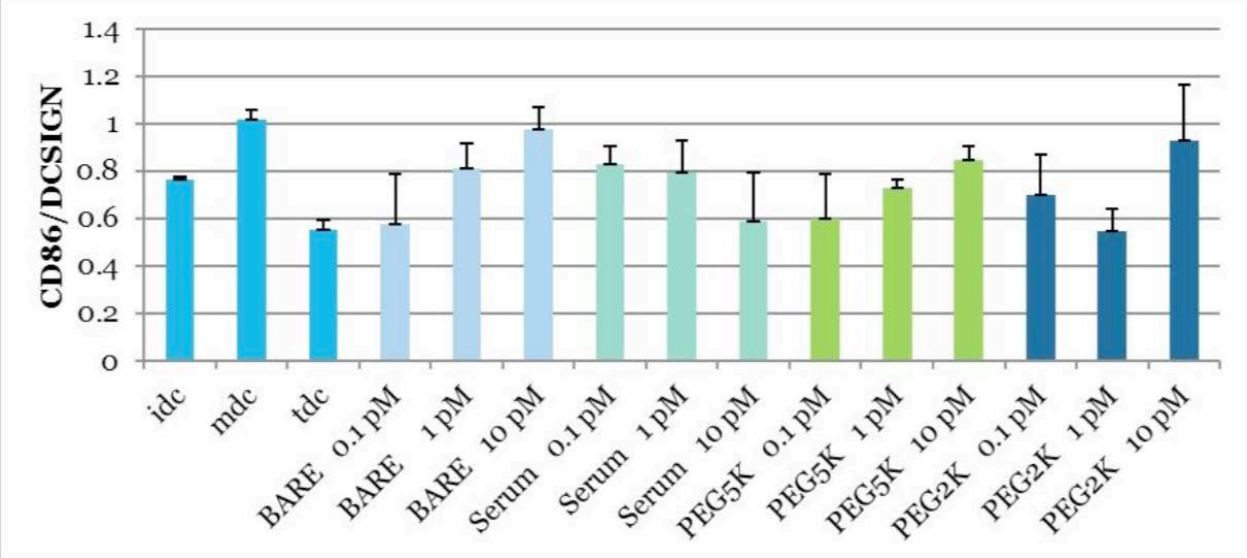
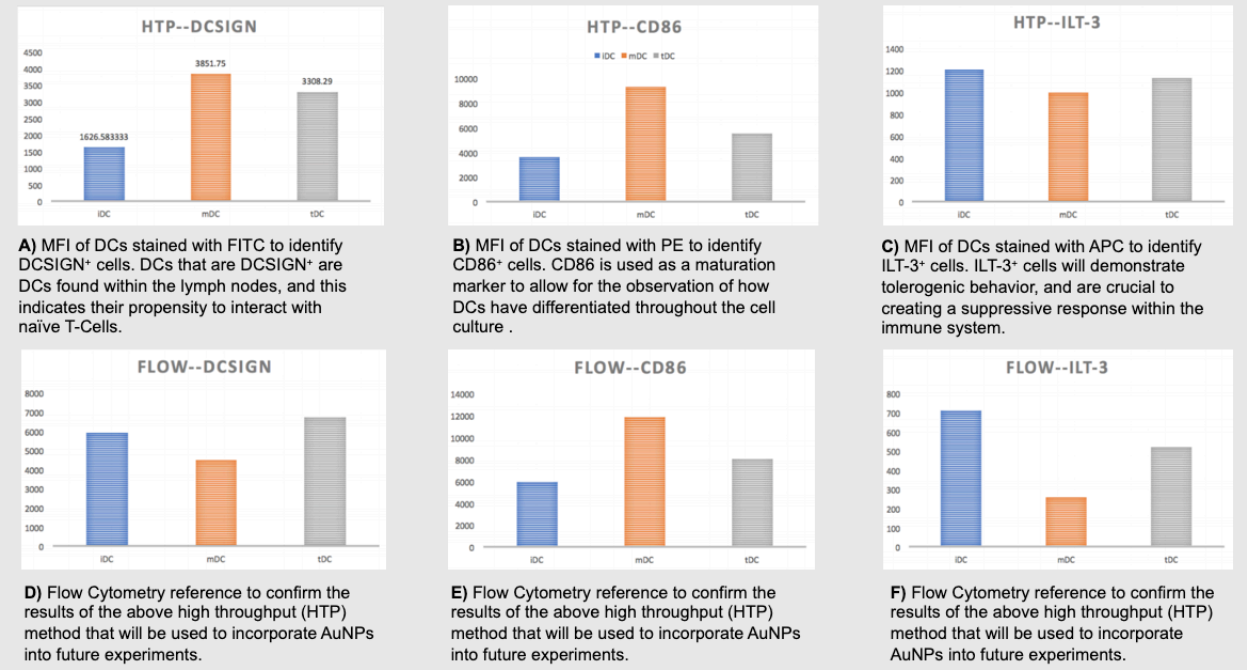


Figure 3. Inflammatory maturation factor (CD86/DCSIGN) of DCs treated with AuNPs with mean ± range, n=2 donors. Treatments of different AuNP coatings (bare, serum, PEG-2K, PEG-5K) and concentrations (0.1 pM, 1.0 pM, 10 pM) were used. DCs were incubated with AuNP treatments for 24 hours at 37°C. DCs were stained with antibodies for surface marker expression, stained with isotype for background and geometric mean fluorescent intensities were determined for CD86 and DCSIGN expression for each treatment group using a Tecan Infinite F500 microplate reader.



A) MFI of DCs stained with FITC to identify DCSIGN⁺ cells. DCs that are DCSIGN⁺ are DCs found within the lymph nodes, and this indicates their propensity to interact with naïve T-Cells.

B) MFI of DCs stained with PE to identify CD86⁺ cells. CD86 is used as a maturation marker to allow for the observation of how DCs have differentiated throughout the cell culture .

C) MFI of DCs stained with APC to identify ILT-3⁺ cells. ILT-3⁺ cells will demonstrate tolerogenic behavior, and are crucial to creating a suppressive response within the immune system.

D) Flow Cytometry reference to confirm the results of the above high throughput (HTP) method that will be used to incorporate AuNPs into future experiments.

E) Flow Cytometry reference to confirm the results of the above high throughput (HTP) method that will be used to incorporate AuNPs into future experiments.

F) Flow Cytometry reference to confirm the results of the above high throughput (HTP) method that will be used to incorporate AuNPs into future experiments.

Figure 4. Confirmation of successful DC culture results based on HTP and flow cytometry methodology. Cell populations cultured under standard techniques demonstrate proper ratios of maturation and tolerization over the course of 5 day culture. A-C demonstrate these results in the HTP format, and D-F confirm these results with higher specificity based on flow cytometry analysis.

Discussion [41]

The phenotype of DCs was differentially modulated by bare, serum-coated, PEG- 2K coated, and PEG-5K coated AuNPs. Specifically, serum coated AuNPs had the highest levels of TMF (ILT3/CD86) for all concentration groups (0.1 pM, 1.0 pM, 10 pM), and all concentrations of serum coated AuNPs were also higher than the TMF for the tDC control. Additionally, the serum coated AuNPs showed concentration dependence for both the TMF and IMF values; increasing the concentrations of serum coated AuNPs increased the TMF and decreased the IMF. This indicates that the interaction between serum coated AuNPs and DCs resulted in greater levels of ILT3 expression on the DCs and a tolerogenic phenotype.

The bare AuNPs also showed a concentration dependent effect but for IMF expression, where increasing bare AuNP concentrations showed an increased IMF. However, the IMFs for

all bare AuNP concentrations were still lower than the positive control, mDC. These results indicate that in the presence of bare AuNPs, DC expression of CD86 increases, therefore causing an inflammatory, activated DC phenotype. The PEG-5K treatment group did not show concentration dependence for TMF, and the TMF values of all concentrations were closest to the iDC control. While concentration dependence was seen for PEG-5K coated AuNPs for IMF levels, these values were also closer to the iDC control than tDCs or mDCs. The PEG-2K coated AuNP treatment stayed within a similar range of TMFs and IMFs as the PEG-5K coated AuNP group. However, a slight concentration dependence was shown for the PEG-2K AuNP treatment for TMF, but not for IMF. These results indicate that the PEG-2K and PEG-5K AuNP coatings did not show a significant change in DC phenotype towards an activating or tolerogenic phenotype.

It is important to note that this data was accumulated from only two donors. More trials are necessary to demonstrate the statistical significance of the trends observed in these preliminary results for bare and serum coated AuNPs, as well as to demonstrate that the PEGylated AuNPs did not have a significant effect on DC phenotype. Additionally, an assumption being made in the analysis of the data in this study is that the AuNPs are actually being phagocytized by the DCs, which may not necessarily be the case. Because this is an *in vitro* study, DCs may simply be interacting with serum molecules and NPs in solution because there is not a complicated matrix of additional molecules and cells that would be found *in vivo*.

Figure 4 demonstrates corrections made to the DC culture setting have had a successful impact and demonstrate proper control results. These results suggested that AuNPs should be

reincorporated into experimentation and that results obtained in future trials will be viable so long as these controls are replicated.

Conclusions and Future Work

The continuation of this research did not generate new results with AuNPs incorporated into trials. The purpose of my contribution to this work was to reinstate successful DC culture into the Babensee Lab protocols with HTP methodology. A standstill was reached with successful DC culture that prevented viable results from being obtained to assess the impact of AuNPs on DCs. Ultimately, successful HTP has been obtained after minor corrections have been made to the culture process.

Furthermore, other undergraduates have been trained to successfully conduct DC culture in Babensee Lab going forward and will be incorporating NPs into future trials in semesters to come. The correction of HTP DC culture will have broad impacts on experimentation that is currently being developed and will allow for expedited production of results regarding the impact of biomaterials on DC populations.

Additionally, a protocol for HTP flow cytometry has been developed for an increase in specificity when observing immunofluorescence. This protocol was developed by Sommer Durham and I and will allow for HTP methodology to be maintained and continue to expedite results, as well as more accurate and repeatable results being obtained to characterize results from biomaterial applications on cell populations. This protocol will have a broad impact on all future work conducted in the Babensee Lab and is an exciting development from my time here.

Future work will include multiplex assays on DC culture supernatants to characterize the microenvironment in which cells have been cultured. This characterization will allow for a highly tuned approach to diseases originating in the immune system and lends to extensive

development of biomaterial applications for further *in vitro* assessment. Additionally, the research conducted in this experiment will hopefully be scaled up to an in vivo model to assess physiological impact that biomaterial treatments can have on immune based diseases. Using the results generated from this study, a murine model for cancer, multiple sclerosis, or HIV/AIDS could be analyzed for benefit from biomaterial treatment. Additionally, drug delivery with the NPs used in this study should be assessed to identify the potential benefit on the aforementioned ailments. This will be possible due to the fact that DC culture has been successfully completed and will continue to be conducted in the Babensee Lab by future undergraduates Ayan Dasgupta and Kalyn Druhot.

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